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Fang, Xiang, Neal L. Weintraub, Christine L. Oltman, Lynn L. Stoll, Terry L. Kaduce, Shawn Harmon, Kevin C. Dellsperger, Christophe Morisseau, Bruce D. Hammock, and Arthur A. Spector. Human coronary endothelial cells convert 14,15-EET to a biologically active chain-shortened epoxide. *Am J Physiol Heart Circ Physiol* 283: H2306–H2314, 2002. First published August 22, 2002; 10.1152/ajpheart.00448.2002.—Cytochrome *P*-450 epoxygenase-derived epoxyeicosatrienoic acids (EETs) play an important role in the regulation of vascular reactivity and function. Conversion to the corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolases is thought to be the major pathway of EET metabolism in mammalian vascular cells. However, when human coronary artery endothelial cells (HCEC) were incubated with ^{3}H-labeled 14,15-EET, chain-shortened epoxy fatty acids, rather than DHET, were the most abundant metabolites. After 4 h of incubation, 23% of the total radioactivity remaining in the medium was converted to 10,11-epoxy-hexadecadienoic acid (16:2), a product formed from 14,15-EET by two cycles of β-oxidation, whereas only 15% was present as 14,15-DHET. Although abundantly present in the medium, 10,11-epoxy-16:2 was not detected in the cell lipids. Exogenously applied ^{3}H-labeled 10,11-epoxy-16:2 was neither metabolized nor retained in the cells, suggesting that 10,11-epoxy-16:2 is a major product of 14,15-EET metabolism in HCEC. 10,11-Epoxy-16:2 produced potent dilation in coronary microvessels. 10,11-Epoxy-16:2 also potently inhibited tumor necrosis factor-α-induced production of IL-8, a proinflammatory cytokine, by HCEC. These findings implicate β-oxidation as a major pathway of 14,15-EET metabolism in HCEC and provide the first evidence that EET-derived chain-shortened epoxy fatty acids are biologically active.

beta-oxidation; vasorelaxation; inflammation; epoxyeicosatrienoic acid

ENDOTHELIUM-DERIVED epoxyeicosatrienoic acids (EETs) produced by arachidonic acid (AA) cytochrome *P*-450 epoxygenases are thought to play an important role in vascular biology. The four EET regioisomers, 5,6-, 8,9-, 11,12-, and 14,15-EET, potently dilate coronary arteries and other blood vessels through activation of maxi-Ca^{2+}-activated K^+ channels (5, 15, 39). Therefore, EETs may function as endothelium-derived hyperpolarizing factors in some vascular beds (4, 13). In addition, EETs modulate a variety of cellular functions and signaling pathways, including protein kinase C (28), Ca^{2+} mobilization (12, 23), tyrosine kinases, mitogen-activated protein kinases, extracellular signal-regulated kinases 1 and 2 (6, 16, 20), cyclooxygenase (11, 14), mono-ADP-ribosylation (22), $G_s\alpha$ protein (26), and expression of adhesion molecules (25). Thus EETs are involved in regulation of vascular function, smooth muscle cell proliferation, and vascular inflammation (5, 15, 39).

The major metabolic fate of EETs in the vasculature is thought to be conversion to the corresponding dihydroxyeicosatrienoic acids (DHETs) catalyzed by epoxide hydrolases, particularly the soluble form of epoxide hydrolase (sEH) (9). Inhibition of sEH by *N,N′*-dicyclohexyl urea (DCU) in rats (37) and sEH knockout in male mice (31) reduced blood pressure. These observations suggest that sEH may play an important role in metabolizing EETs in the vasculature and, consequently, in regulating vascular function. On the other hand, EET metabolism can also be modulated by fatty acid binding proteins, cyclooxygenase, and cytosolic glutathione *S*-transferases (5, 39). Moreover, when sEH was inhibited with DCU in porcine coronary artery endothelial cells, we observed (9) the emergence of a novel β-oxidation pathway that converts EETs to chain-shortened epoxy fatty acid derivatives. These studies suggest that enzymatic pathways other than sEH could also play an important role in regulating the metabolism and bioactivity of EETs in the vasculature.

Most of the studies of EET metabolism in vascular cells have been performed in cultured porcine cells (9, 10, 33–35), and the relative importance of sEH vs. β-oxidation or other pathways of EET metabolism in human arterial endothelial cells is unknown. In addition, whether the epoxy fatty acid derivatives produced through β-oxidation possess biological activity is not

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known. Because DHETs and 7,8-dihydroxy-hexadecadienoic acid, a DHET β-oxidation product, are capable of producing vasodilation (10, 35), we considered it possible that the chain-shortened epoxy fatty acids may also have biological activity.

The purpose of the present study was to delineate the pathways of EET metabolism in human coronary artery endothelial cells (HCEC) and to determine whether any novel metabolites that are formed have biological activities. Our studies focused on 14,15-EET because this EET regioisomer is abundantly produced by coronary endothelial cells stimulated with bradykinin or methacholine and possesses important bioactivities in the coronary circulation (1, 24, 35). We found that β-oxidation products are the most abundant EET metabolites produced by the human coronary cells in culture. In addition, the most abundant β-oxidation product of 14,15-EET, 10,11-epoxy-hexadecadienoic acid (16:2), was found to produce potent vasodilation of coronary microvessels and to have anti-inflammatory activity. These findings suggest that chain-shortened epoxy fatty acids may contribute to the dilatory and anti-inflammatory actions of EETs in the vasculature.

MATERIALS AND METHODS

Cell culture and incubation. HCEC were purchased from Clonetics Cell Discovery Systems (Walkersville, MD) and grown in Clonetics EGM-2 MV medium containing 10% FBS. The cultures were maintained until confluent at 37°C in a humidified atmosphere containing 5% CO_2. Stocks were subcultured weekly by trypsinization, and cultures were used for experiments between *passages 7* and *9*. Experiments were carried out with confluent monolayers and EGM-2 MV medium containing 0.1 μM BSA. Porcine coronary artery endothelial cells (PCEC) were isolated and grown in modified medium 199 supplemented with 10% FBS as described previously (34). For comparison, human umbilical vein endothelial cells (HUVEC) and human coronary artery smooth muscle cells (HCSMC) were also tested in some experiments.

^{3}H-labeled 14,15-EET was synthesized as described previously (9). [^{3}H]14,15-EET was mixed with the corresponding nonradiolabeled compound to obtain the substrate concentration necessary for each experiment at a specific activity of 0.1 μCi/nmol. Cells were incubated with radiolabeled substrate in medium EGM-2 MV for the indicated times. After incubation, the medium was collected and the cells were harvested by scraping into methanol.

Assay of incubation medium. The radioactivity remaining in the medium after the incubation was measured by liquid scintillation counting. Lipids contained in the medium were extracted and analyzed by reverse-phase HPLC. The elution profile consisted of water adjusted to pH 4.0 with formic acid and an acetonitrile gradient that increased from 30% to 57% acetonitrile over 25 min, from 57% to 65% over 15 min, and then was held at 65% for 5 min, after which time the acetonitrile was taken to 100% and held there for 15 min. The distribution of radioactivity was measured by combining the column effluent with scintillator solution and passing the mixture through an on-line flow detector (9).

Analyses of cell lipids. Lipids were extracted from the cells with chloroform-methanol. After the phases were separated and the solvent was removed under N_2 the lipids were dissolved in chloroform-methanol, and an aliquot of this mixture was dried under N_2 and assayed for radioactivity in a liquid scintillation spectrometer (10). To separate and identify the radiolabeled fatty acid derivatives incorporated into the cell lipids, additional aliquots of the extract were hydrolyzed for 1 h at 50°C in 0.5 ml of methanol containing 50 μl of 0.2 N NaOH and 10% H_2O. After the pH was adjusted to 7.2, the free fatty acids were extracted and separated by HPLC (9).

Identification of EET metabolites. The structure of lipid metabolites contained in the medium and cell lipids was identified with a Hewlett-Packard 1100 MSD liquid chromotography/mass spectroscopy (LC/MS) system (2, 9). HPLC separation was carried out with a C_{18} column and mobile phase solvents consisting of H_2O-formic acid (100:0.03, vol/vol; *solvent A*) and acetonitrile (*solvent B*) at a flow rate of 0.7 ml/min. The gradient was maintained at 30% *solvent B* for the first 2 min and then linearly ramped to 57% *solvent B* at 20 min, 65% *solvent B* at 40 min, 70% *solvent B* at 45 min, and 95% *solvent B* at 50 min. Negative-ion electrospray was used with the fragmentor voltage set at 140 V to produce in-source collision-induced decompositions (CID). N_2 nebulizing gas was maintained at 60 bars, whereas the N_2 drying gas was set to a flow rate of 10 l/min at 350°C. Data were processed with the Hewlett-Packard Chemstation software program.

Determination of sEH activity. Cells were harvested and suspended in 1 ml of chilled 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, phenylmethylsulfonyl fluoride, and dithiothreitol. The cells were disrupted with a Polytron homogenizer at 9,000 rpm for 30 s, the homogenate was centrifuged at 9,000 *g* for 10 min at 4°C, and the supernatant solution was used as the enzyme extract. Epoxide hydrolase activity was measured with racemic *trans*-[^{3}H]-1,3-diphenylpropene oxide (9).

Determination of vasorelaxation in coronary microvessels. An isolated, pressurized microvascular preparation was used to study porcine and human coronary microvessels (27). Briefly, porcine coronary microvessels (102 ± 7-μm inside diameter and 1–1.5 mm long) were dissected and transferred to an organ chamber. The chamber was placed on the stage of an inverted microscope equipped with a video camera, a monitor, and a calibrated video caliper. Microvessels were pressurized to 60 cmH_2O under no-flow conditions. Oxygenated (20% O_2-5% CO_2, balance N_2), warmed (37°C) Krebs solution was continuously circulated through the organ chamber. The chamber was then rinsed with fresh Krebs solution, and the vessels were submaximally (30–60%) preconstricted with endothelin (0.2–0.9 nM) or isotonic KCl (50 mM, prepared by substituting an equimolar amount of KCl for NaCl). Cumulative concentration-response curves were generated for 10,11-epoxy-16:2 and, for comparison, 14,15-EET by adding these compounds to the circulating bath. A similar procedure was used to test the effect of 10,11-epoxy-16:2 on human coronary microvessels. Experimental protocols for the human tissue study were approved by the appropriate institutional review committee and meet the guidelines of the responsible governmental agency.

Effect of EET metabolites on TNF-α-induced IL-8 release. HCEC were grown to confluence in 48-well plates that had been precoated with 0.1% gelatin. Cells were incubated for 24 h with MEM containing 2% FBS, 0, 1, or 10 ng/ml TNF-α, and the indicated concentration of lipid. At the end of the 24-h incubation, the media were collected and the concentration of IL-8 was determined by ELISA. Briefly, 96-well Nunc Immuno plates were coated overnight with monoclonal antibody against IL-8. The plates were blocked for 1 h with phosphate-buffered saline containing 1% BSA, 5% sucrose, and 0.05% sodium azide. Samples were diluted in the appropriate medium and added to the wells with 40 ng/ml biotin-

ylated goat anti-human IL-8 polyclonal antibody. Antibody binding was visualized with horseradish peroxidase-conjugated streptavidin (1:1,000; Pierce) and TMB liquid substrate system. The reaction was stopped by addition of 0.5 M H_2SO_4, and the absorbance was measured at 450 nm. Values were determined relative to a standard curve.

Statistical analysis. The data are expressed as means ± SE. Values were analyzed by Student's *t*-tests for unpaired data or by one-way analysis of variance followed by Fisher's exact test. Probability values of 0.05 or less were considered to be statistically significant.

RESULTS

Metabolism of 14,15-EET by human and porcine coronary endothelial cells. HCEC cultures were incubated with radiolabeled 14,15-EET to investigate its metabolism. PCEC cultures were studied in parallel for comparison. HCEC and PCEC had a similar capacity to take up [^{3}H]14,15-EET. After a 75-min incubation with 2 μM [^{3}H]14,15-EET, 14% and 12% of total radioactivity were present in the HCEC and PCEC lipids, respectively. When the PCEC were incubated with 2 μM [^{3}H]14,15-EET for 4 h, 90% of the radioactivity present in the medium was converted to a single metabolite that coeluted with 14,15-DHET (Fig. 1 *A*). This result is consistent with our previous observations (9). However, when the HCEC were incubated with 14,15-EET under identical conditions, only 15% of the radioactivity in the medium was converted to 14,15-DHET (Fig. 1*B*). Two additional major radiolabeled products were detected, with retention times of 40 min (*product A*) and 31 min (*product B*). *Products A* and *B* accounted for 5% and 23% of total radioactivity in the medium, respectively.

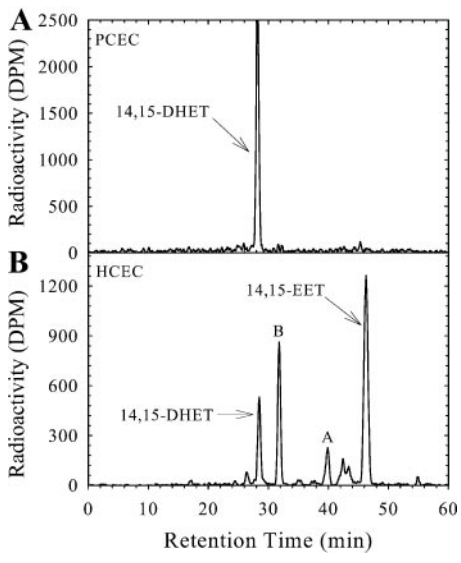

Fig. 1. Formation of ^{3}H-labeled 14,15-epoxyeicosatrienoic acid (EET) metabolites by porcine coronary artery endothelial cells (PCEC; *A*) and human coronary endothelial cells (HCEC; *B*). Cells were incubated with 2 μM [^{3}H]14,15-EET for 4 h. The incubation media were removed, and lipids were extracted and analyzed for radioactivity by HPLC with an on-line flow scintillation counter. Radiochromatograms from a single culture are shown, but similar results were obtained from 2 additional cultures in each case. DHET, dihydroxyeicosatrienoic acid.

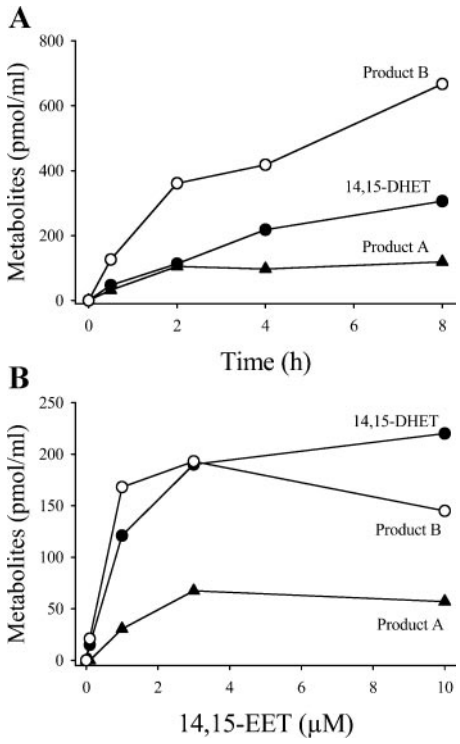

Fig. 2. Time- and concentration-dependent formation of chain-shortened epoxy fatty acids in the culture medium. HCEC were incubated with 2 μM [^{3}H]14,15-EET for various times (*A*) or for 4 h with medium containing different amounts of [^{3}H]14,15-EET (*B*). After incubation, the medium was assayed for radioactivity. Values are calculated from the specific activity of [^{3}H]14,15-EET added to the cultures. Each point is the average of results obtained from 2 separate cultures, and both values were agreed within 10%.

Products A and *B* and 14,15-DHET were detected in the medium as early as 30 min when the HCEC were incubated with [^{3}H]14,15-EET. The metabolites accumulated in the medium in a time-dependent manner (Fig. 2*A*). After an 8-h incubation with 2 μM [^{3}H]14,15-EET, *products A* and *B* accounted for 8% and 46% of the total medium radioactivity, respectively, whereas 14,15-DHET accounted for 19%. These results suggest that an enzymatic pathway other than sEH is primarily responsible for the metabolism of 14,15-EET in HCEC. The dependence of radiolabeled metabolite formation on the concentration of [^{3}H]14,15-EET added to the incubation medium in a 4-h incubation is illustrated in Fig. 2*B*. The amount of *product B* relative to DHET was higher when the initial 14,15-EET concentration was 1 μM. However, formation of 14,15-DHET exceeded that of *product B* when the cells were incubated with 10 μM 14,15-EET. Estimated K_m and V_{max} values from a plot of $1/V_0$ vs. $1/[S]$ (V_0, initial rate; [S], substrate concentration) according to the Michaelis-Menten equation were 2.2 μM and 1.4 pmol/min for 14,15-DHET and 1.08 μM and 1.04 pmol/min for *product B*.

Identification of products A and B. To identify the chemical structure of the unknown compounds, the products contained in the incubation medium were methylated to derivatize the carboxyl groups and then

the products were exposed to conditions of acetylation (Table 1). *Products A* and *B*, as well as 14,15-EET, were methylated; however, the resulting methyl esters could not be acetylated. Methylation of 14,15-EET resulted in an increase in the retention time (RT) by 10.8 min, but attempted acetylation of the methyl ester did not further increase the RT. Likewise, the RT increased by 15.5 and 16.2 min, respectively, after methylation of *products A* and *B*. However, the RT did not further increase when the methyl esters of these products were exposed to conditions of acetylation. In contrast, the product that coeluted with 14,15-DHET and an authentic 14,15-DHET standard were methylated and acetylated. Methylation of this product and the 14,15-DHET standard increased the RT by 11.8 min, and acetylation of the methyl esters further increased the RT by 8.2 min. These results indicate that *products A* and *B* contain carboxyl groups but not hydroxyl groups, confirming that they are not DHET derivatives.

The major radiolabeled compounds that accumulated in the medium were identified by LC/MS. In-source CID was utilized to generate additional structural information. *Product A* (Fig. 3*A*) yielded a base peak of mass-to-charge ratio (*m/z*) 293 (carboxylate anion) and fragments of *m/z* 275 (loss of H_2O), 249 (loss of CO_2), and 193 (cleavage of the oxirane ring). This is consistent with a structure of 12,13-epoxyoctadecadienoic acid (12,13-epoxy-18:2). *Product B* (Fig. 3*B*) yielded a carboxylate base peak of *m/z* 265, and fragments of *m/z* 247 (loss of H_2O), 203 (loss of $H_2O + CO_2$), and 165 (oxirane ring cleavage). This is consistent with a structure of 10,11-epoxy-16:2.

These findings indicate that under basal conditions (i.e., in the absence of sEH inhibition), HCEC actively convert 14,15-EET to chain-shortened epoxy fatty acids through β-oxidation, with each cycle removing two carbons from the carboxyl end of the molecule. Thus β-oxidation appears to be the major metabolic pathway of 14,15-EET in HCEC cultures.

Determination of sEH activity. To investigate the mechanism responsible for the difference in the amount of EET converted to DHET in PCEC and HCEC, we compared the enzymatic activity of sEH in

Table 1. *Chemical derivatization of 14,15-EET metabolites contained in incubation medium*

Radiolabeled Compound	Retention Time, min		
	Unmodified	After methylation reaction	After methylation and acetylation reaction
14,15-EET	46.3	57.1	57.1
Product A	40.0	55.5	55.5
Product B	32.0	48.2	48.2
14,15-DHET	28.4	40.2	48.4

Human coronary endothelial cells were incubated with 2 μM [^{3}H]14,15-epoxyeicosatrienoic acid (EET) for 4 h. The radiolabeled products contained in the medium were extracted, and an aliquot was assayed by HPLC. The remainder was methylated, and an aliquot was analyzed by reverse-phase HPLC with the on-line flow scintillation counter. Another aliquot of the methylated products was exposed to the conditions of acetylation and then analyzed by HPLC as above. DHET, dihydroxyeicosatrienoic acid.

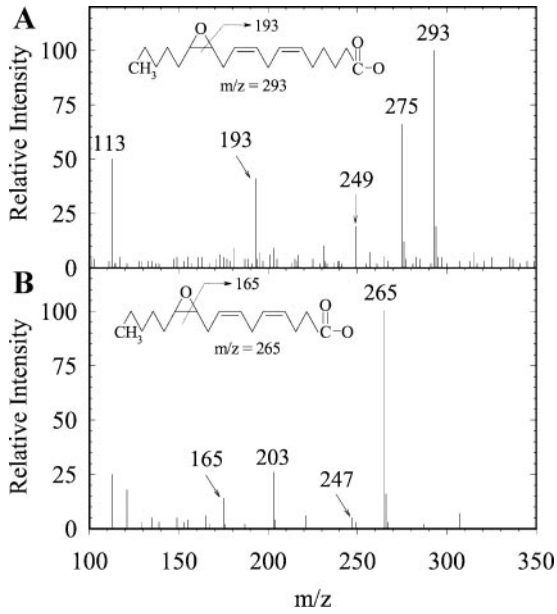

Fig. 3. Mass spectra of *product A* (*A*) and *product B* (*B*). The spectra were obtained by liquid chromotography/mass spectroscopy with in-source collision-induced decompositions. To obtain sufficient quantities of products for structural identification, it was necessary to use 75-cm^2 HCEC cultures. HPLC analysis indicated that the pattern of metabolite formation by the 75-cm^2 cultures was similar to that shown in Fig. 1 (*B*). The spectra are consistent with a structure of 12,13-epoxyoctadecadienoic acid (18:2) (*A*) and 10,11-epoxy-hexadecadienoic acid (16:2) (*B*). *m/z*, Mass-to-charge ratio.

PCEC and HCEC. The sEH activity in cellular homogenates was 30-fold higher in PCEC compared with HCEC [524 ± 57 (PCEC) vs. 16.5 ± 1.0 (HCEC) pmol·min^{-1}·mg protein^{-1}; $n = 4$, $P < 0.01$]. This difference in sEH activity between PCEC and HCEC is consistent with the findings obtained in intact cells, in which the conversion of [^{3}H]14,15-EET to [^{3}H]14,15-DHET was substantially greater in PCEC compared with HCEC. Furthermore, a very small amount of sEH protein was detected with a specific sEH antibody against human sEH (data not shown), and this correlates with the low sEH activity in HCEC. The sEH protein in PCEC also was assayed with the specific antibody against human sEH. The human sEH antibody cross-reacted with the porcine sEH, but an intense band with a slightly higher molecular weight than the human sEH protein also was detected in the porcine cell extract. Therefore, we were not able to quantitatively compare the difference in sEH protein between PCEC and HCEC.

Analysis of cell lipids. We next investigated whether the chain-shortened epoxy fatty acids are retained in the cell lipids. After a 4-h incubation with [^{3}H]14,15-EET, ~14% of total radioactivity was present in cell lipids. HPLC analysis of the hydrolyzed cell lipids indicated that 78% of the cell-associated radioactivity was present as 14,15-EET, whereas 8% of the radioactivity was present as a less polar metabolite with RT of 54 min (Fig. 4). This metabolite contained a base peak of *m/z* 347 (carboxylate anion) and fragments of *m/z* 329 (loss of H_2O) and 247 (cleavage in the oxirane ring). This is consistent with the structure of 16,17-

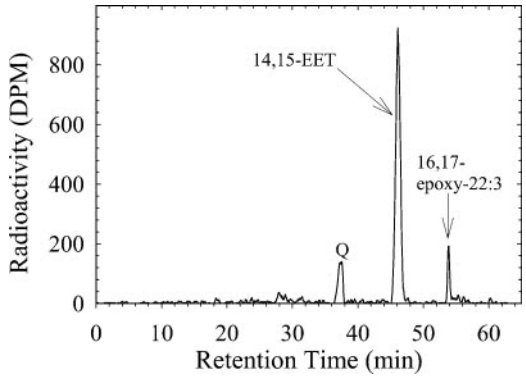

Fig. 4. Radiolabeled 14,15-EET products present in the cell lipids. After incubation of HCEC with 2 μM [^{3}H]14,15-EET as described in Fig. 1, the cell lipids were extracted, hydrolyzed by saponification, and analyzed for radioactivity by reverse-phase HPLC. Radiochromatograms from a single culture are shown, but similar results were obtained from 2 additional cultures in both cases. *Metabolite Q* has not been identified. 22:3, Epoxydocosatrienoic acid.

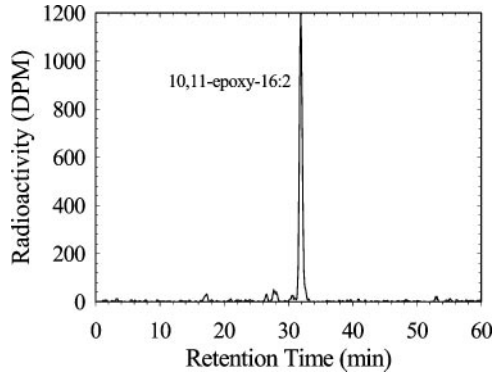

Fig. 5. HPLC analysis of radiolabeled lipids in the medium of HCEC incubated with [^{3}H]10,11-epoxy-16:2. HCEC were incubated with 2 μM [^{3}H]10,11-epoxy-16:2 for 4 h, after which the lipids contained in the medium were extracted and analyzed for radioactivity by HPLC as described in Fig. 1. Results shown are representative of radiochromatograms from 3 separate cultures.

epoxydocosatrienoic acid (16,17-epoxy-22:3), an elongated epoxy fatty acid formed from 14,15-EET (data not shown). 16,17-Epoxy-22:3 was previously identified in PCEC lipids after incubation with [^{3}H]14,15-EET (9). Another compound with a RT of 37.3 min (*product Q*) contained ~11% of total radioactivity, but the structure of this compound has not been determined. Neither 12,13-epoxy-18:2 nor 10,11-epoxy-16:2 was detected in the cell lipids. These findings suggest that the chain-shortened epoxy fatty acids formed through β-oxidation are preferentially released into the extracellular medium by the cells.

Metabolism of 10,11-epoxy-16:2. To investigate the mechanism of 10,11-epoxy-16:2 accumulation in the medium, we determined whether HCEC could incorporate or further metabolize this compound. [^{3}H]10,11-epoxy-16:2 was generated by incubation of [^{3}H]14,15-EET with human aortic smooth muscle cells in the presence of the selective sEH inhibitor DCU, which blocked the formation of 14,15-DHET and increased the amount of [^{3}H]10,11-epoxy-16:2 recovered in the incubation medium (data not shown). The 10,11-epoxy-16:2 was purified by HPLC, and its structure was confirmed by LC/MS. HCEC were then incubated with 2 μM [^{3}H]10,11-epoxy-16:2, and medium- and cell-associated lipids were extracted and analyzed. After 4-h incubation, ~95% of the total radioactivity was recovered in the medium and <5% was present in the cell-associated lipids. The amount of radioactivity in the cell lipids was insufficient to permit analysis by HPLC. However, HPLC analysis of the medium extract showed that 93% of radioactivity was present as 10,11-epoxy-16:2 (Fig. 5), suggesting that HCEC do not avidly metabolize exogenously applied 10,11-epoxy-16:2. Because the cells did not further metabolize 10,11-epoxy-16:2 and incorporated only very small amounts into cell lipids, these results suggest that the compound is a major product of 14,15-EET metabolism.

Vasorelaxant effects of 10,11-epoxy-16:2. Because 14,15-EET, the parent compound of 10,11-epoxy-16:2, is a potent dilator of coronary microvessels (27), we examined whether 10,11-epoxy-16:2 also has vasodilator activity. Like 14,15-EET, 10,11-epoxy-16:2 produced potent dose-dependent relaxation of porcine coronary microvessels that were preconstricted with endothelin [EC_{50} expressed as −log[M]: 12.3 ± 0.6 (10,11-epoxy-16:2) vs. 12.5 ± 0.4 (14,15-EET)] (Fig. 6*A*). Moreover, 10,11-epoxy-16:2 also potently dilated human coronary microvessels preconstricted with endothelin (EC_{50} expressed as −log[M]: 12.07 ± 0.5; n = 4) (Fig. 6*B*). However, neither 10,11-epoxy-16:2 nor

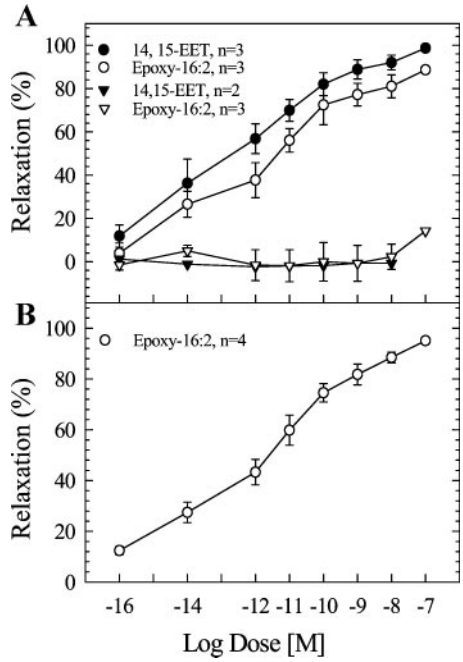

Fig. 6. Vasoactive effects of 10,11-epoxy-16:2 in porcine (*A*) and human (*B*) coronary microvessels. Isolated microvessels were preconstricted with endothelin (circles) or KCl (triangles) and then exposed to increasing concentrations of 14,15-EET (closed symbols) or 10,11-epoxy-16:2 (open symbols). Vessel diameter was monitored with a video camera, and results are expressed as %relaxation from the preconstricted diameter. Values are means ± SE; n = no. of separate preparations tested.

14,15-EET relaxed porcine coronary microvessels preconstricted with KCl (Fig. 6*A*), suggesting a hyperpolarization-dependent mechanism of action as demonstrated for 14,15-EET in coronary microvessels from other species (4, 27).

Effect of 10,11-epoxy-16:2 on TNF-α-induced IL-8 release. Because EETs possess anti-inflammatory properties that are independent of their membrane-hyperpolarizing effects (25), we determined whether 10,11-epoxy-16:2 also has anti-inflammatory effects. HCEC were stimulated with TNF-α in the presence or absence of AA, 14,15-EET, or 10,11-epoxy 16:2 for 24 h, and the incubation media were assayed for IL-8 by ELISA. TNF-α induced IL-8 release in a dose-dependent manner (data not shown). TNF-α (1 ng/ml) produced a 25-fold increase in IL-8 release, and this was inhibited by 14,15-EET (0.3 μM) but not by AA at the same concentration (Fig. 7 *A*). The inhibitory effect of 14,15-EET on TNF-α (1 ng/ml)-induced IL-8 release was dose dependent. Similarly, 10,11-epoxy-16:2 produced dose-dependent inhibition of IL-8 release (Fig. 7*B*). Thus, in addition to having potent vasodilating effects, 10,11-epoxy-16:2 inhibits inflammatory activation of HCEC.

Metabolism of 14,15-EET by other cultured human vascular cells. We investigated whether other types of human vascular cells can also produce chain-shortened

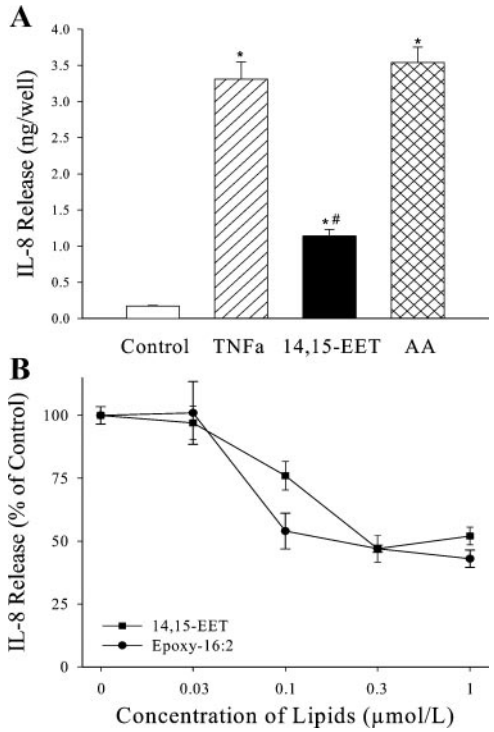

Fig. 7. Effect of 10,11-epoxy-16:2 on TNF-α-induced IL-8 release from HCEC. Confluent HCEC were incubated for 24 h with MEM containing 2% FBS with no TNF-α (control) or TNF-α along with 0.3 μM arachidonic acid (AA) or 0.3 μM 14,15-EET (*A*) or the indicated concentration of 14,15-EET or 10,11-epoxy 16:2 (*B*). The concentration of TNF-α used was 1 ng/ml in both *A* and *B*. At the end of the 24-h incubation, the media were collected and assayed for IL-8 by ELISA. Data are means ± SE ($n = 4$). *$P < 0.05$ vs. control; #$P < 0.05$ vs. TNF-α group.

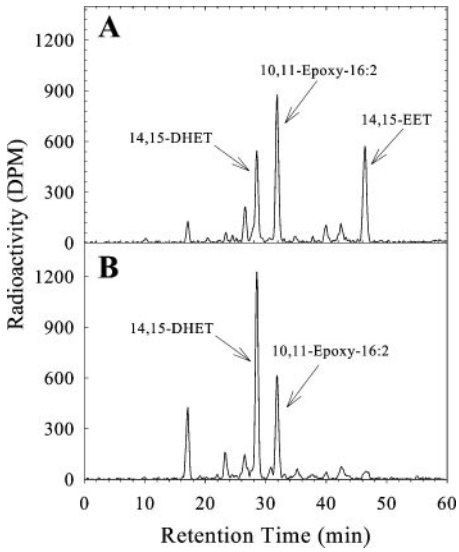

Fig. 8. Formation of [^{3}H]14,15-EET metabolites by human coronary artery smooth muscle cells (*A*) and human umbilical vein endothelial cells (*B*). Cells were incubated with 2 μM [^{3}H]14,15-EET in ECM-2 medium containing 0.1 μM BSA. After 4 h of incubation, the media were removed and the lipids were extracted and analyzed for radiolabeled metabolites as described in Fig. 1. Radiochromatograms from a single culture are shown, but similar results were obtained from 2 additional cultures in each case.

epoxy fatty acids. HCSMC and HUVEC were incubated with [^{3}H]14,15-EET. As observed with HCEC, 10,11-epoxy-16:2 was the most abundant radiolabeled metabolite produced by HCSMC. After 4 h of incubation with 2 μM [^{3}H]14,15-EET, 26% of radioactivity remaining in the medium was present as 10,11-epoxy-16:2, 18% was present as 14,15-DHET, and 36% remained as 14,15-EET (Fig. 8*A*). Substantial amounts of 10,11-epoxy-16:2 were also produced from 14,15-EET by HUVEC, although 14,15-DHET was the major metabolite produced by these human cells. After 4 h of incubation, no [^{3}H]14,15-EET was left in the medium, 40% of total radioactivity in the medium was present as 14,15-DHET, and 21% was present as 10,11-epoxy-16:2 (Fig. 8*B*). Several other more polar metabolites also were detected in the HUVEC medium, but they have not been identified. These results indicate that cultured human vascular cells have substantial capacity to produce chain-shortened epoxy fatty acids through a β-oxidation pathway even when sEH is not inhibited. The β-oxidation pathway appears to be particularly important in human coronary artery endothelial and smooth muscle cells.

DISCUSSION

In the present study, we found that 14,15-EET is converted to diol, chain-shortened and -elongated products. However, 10,11-epoxy-16:2, a β-oxidation product, is the most abundant metabolite of [^{3}H]14,15-EET produced by HCEC under most conditions. The cultures did not further metabolize 10,11-epoxy-16:2 or incorporate it into cell lipids, suggesting that 10,11-epoxy-16:2 is a major product of 14,15-EET metabolism

in HCEC. Studies in coronary microvessels indicate that 10,11-epoxy-16:2 is a potent vasodilator and 10,11-epoxy-16:2 also inhibited cytokine-induced IL-8 production by HCEC. These results identify β-oxidation as a major pathway of EET metabolism in HCEC. Moreover, they suggest that the main chain-shortened epoxy fatty acid product derived through β-oxidation can modulate vasoreactivity and inflammation in the coronary circulation.

We previously observed (10, 11, 33–35) that conversion of EETs to DHETs by sEH is the prevailing pathway of EET metabolism in porcine endothelial and smooth muscle cells. Although PCEC were able to produce the chain-shortened epoxy fatty acids, appreciable amounts were formed only when sEH was inhibited (9). These differences in EET metabolism between porcine and human endothelial cells most likely result from differences in the relative activities of sEH, as indicated by our observation that sEH activity is 30-fold higher in PCEC than in HCEC. Low sEH activity is correlated with the presence of a very small amount of immunoreactive sEH protein in HCEC. The enzymes responsible for the β-oxidation of EETs in PCEC and HCEC have not been identified. However, we previously observed (8, 18) that human skin fibroblasts have the capacity to produce chain-shortened fatty acids from EETs and AA through peroxisomal β-oxidation. These observations suggest that peroxisomal β-oxidation also may play an important role in metabolism of EETs in the HCEC. In this regard, chain-shortened fatty acids are produced from hydroxyeicosatetraenoic acids and hydroxyoctadecadienoic acids through β-oxidation by endothelial cells (7, 30), and we have detected the expression of acyl CoA oxidase mRNA, a key enzyme involved in peroxisomal β-oxidation, in HCEC (unpublished observations). The present data demonstrate that β-oxidation also plays a prominent role in metabolism of 14,15-EET in cultured HCSMC and is active in cultured HUVEC. Together with the previous results in human skin fibroblasts (8), these results suggest that peroxisomal β-oxidation may be of major importance in EET metabolism in human cells of both vascular and nonvascular origin. However, whether formation of chain-shortened epoxy fatty acids also occurs in intact human vessels in vivo remains to be determined.

Analysis of the incubation medium after treatment of HCEC with [^{3}H]14,15-EET indicated that the chain-shortened epoxy fatty acid products were not converted to detectable amounts of their corresponding diols. Furthermore, when the cells were exposed to exogenous 10,11-epoxy-16:2, diol metabolites were not detected in the medium even after 4 h of incubation. Thus, compared with 14,15-EET, the 16-carbon epoxide metabolite does not appear to be an effective substrate for the relatively small amount of sEH contained in HCEC. sEH has been shown to exhibit both regio- and enantiomeric selectivity for EETs. For example, the rate of epoxide hydrolysis was found to be threefold greater for 14,15-EET compared with 8,9-EET (38). In addition, varying the carbon chain length altered the capacity of epoxide compounds to induce sEH-dependent cytotoxicity (19). Very little of the exogenously applied 10,11-epoxy-16:2 was incorporated into cell lipids, and the exogenously applied compound did not undergo β-oxidation. This suggests that the 16-carbon epoxy fatty acid is not readily taken up by the endothelial cells. The failure of the cells to take up epoxy-16:2 may also explain why the diol of epoxy-16:2 is not formed by sEH. Therefore, 10,11-epoxy-16:2 appears to be a major product of 14,15-EET metabolism in HCEC.

The present findings are the first demonstration that a chain-shortened epoxy fatty acid produced from EET is biologically active. Similar to 14,15-EET, 10,11-epoxy-16:2 dilated coronary microvessels constricted with endothelin, but not KCl, suggesting a hyperpolarizing effect on smooth muscle cells. Because appreciable amounts of 10,11-epoxy-16:2 are not taken up or further metabolized by the cells, incorporation of the compound into membrane phospholipid domains or conversion to a metabolic product does not appear to be required for vasorelaxation. Recent evidence suggests that the biological activity of 14,15-EET is mediated by a receptor present in the cell surface (32, 36). However, whether the effect is due to a direct interaction of 10,11-epoxy-16:2 with a cell membrane receptor or an indirect effect of binding to other cellular structures remains to be determined. Considering the potency of 10,11-epoxy-16:2 to induce vasorelaxation, it is possible that the compound could play a role in the vasoregulation of the coronary circulation. Because epoxy-16:2 was not retained in cell lipids, however, it would most likely not be capable of potentiating agonist-induced activation of endothelium-dependent relaxation, as has been demonstrated for EETs (34). Because DHETs also cause potent vasodilation in canine coronary microcirculation (27), it appears that both sEH and β-oxidation may modulate the vasoactivity of EETs in coronary microcirculation. However, the relative contribution of these EET pathways in regulating vasoactivity and signaling mechanisms remains to be determined.

Physiological concentrations of EETs inhibit cytokine-induced endothelial cell adhesion molecule expression and prevent leukocyte adhesion to the vascular wall (25). IL-8 represents an important proinflammatory cytokine that was recently demonstrated to play a major role in the development of atherosclerosis in mice (21, 29). It is produced by endothelial cells in response to inflammatory mediators and is chemotactic for neutrophils and T lymphocytes, which are prevalent in the fibrous cap of atherosclerotic lesions. IL-8 induces chemotaxis of freshly isolated peripheral blood monocytes and converts monocyte rolling to firm adhesion on endothelial monolayers (17). Furthermore, LDL receptor −/− mice that were irradiated and repopulated with bone marrow cells lacking the murine homolog of CXCR-2, the principal receptor for IL-8, had less extensive atherosclerotic lesions and fewer infiltrating macrophages compared with mice receiving bone marrow cells that express CXCR-2 (3). Previous studies found that 11,12-EET, but not 14,15-EET, in-

hibited TNF-α- and IL-1α-induced expression of vascular cell adhesion molecule 1 (25). Our current observations indicate that 14,15-EET also inhibits TNF-α-induced IL-8 release from HCEC. Furthermore, like 14,15-EET, 10,11-epoxy-16:2 possesses potent anti-inflammatory effects and, therefore, might be protective against the development of atherosclerosis. Because it is not clear whether 10,11-epoxy-16:2 can be made by human vessels in vivo, however, the physiological relevance of these findings remains to be determined.

In summary, these findings suggest that formation of novel chain-shortened fatty acid epoxides through β-oxidation is a major pathway of 14,15-EET metabolism in cultured HCEC. The main product, a 16-carbon epoxy fatty acid, is released from the cells, suggesting that it is a major product of 14,15-EET metabolism. We have identified 10,11-epoxy-16:2 as a potent vasodilator and anti-inflammatory mediator in the coronary circulation, demonstrating for the first time that a chain-shortened epoxy fatty acid formed from an EET is biologically active. These findings could have important implications with regard to the metabolism of EETs in the human coronary circulation and their regulation of vascular function.

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REFERENCES

1. **Baron A, Frieden M, and Beny JL.** Epoxyeicosatrienoic acids activate a high-conductance, Ca^{2+}-dependent K^+ channel on pig coronary artery endothelial cells. *J Physiol* 504: 537–543, 1997.
2. **Bernstrom K, Kayganich K, and Murphy RC.** Collisionally induced dissociation of epoxyeicosatrienoic acids and epoxyeicosatrienoic acid-phospholipid molecular species. *Anal Biochem* 198: 203–211, 1991.
3. **Boisvert WA, Santiago R, Curtiss LK, and Terkeltaub RA.** A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* 101: 353–363, 1998.
4. **Campbell WB, Gebremedhin D, Pratt PF, and Harder DR.** Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 78: 415–423, 1996.
5. **Capdevila JH, Falck JR, and Harris RC.** Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* 41: 163–181, 2000.
6. **Chen JK, Wang DW, Falck JR, Capdevila J, and Harris RC.** Transfection of an active cytochrome P450 arachidonic acid epoxygenase indicates that 14,15-epoxyeicosatrienoic acid functions as an intracellular second messenger in response to epidermal growth factor. *J Biol Chem* 274: 4764–4769, 1999.
7. **Fang X, Kaduce TL, and Spector AA.** 13-(S)-hydroxyoctadecadienoic acid (13-HODE) incorporation and conversion to novel products by endothelial cells. *J Lipid Res* 40: 699–707, 1999.
8. **Fang X, Kaduce TL, VanRollins M, Weintraub NL, and Spector AA.** Conversion of epoxyeicosatrienoic acids (EETs) to chain-shortened epoxy fatty acids by human skin fibroblasts. *J Lipid Res* 41: 66–74, 2000.
9. **Fang X, Kaduce TL, Weintraub NL, Harmon S, Teesch LM, Morisseau C, Thompson DA, Hammock BD, and Spector AA.** Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition. *J Biol Chem* 276: 14867–14874, 2001.
10. **Fang X, Kaduce TL, Weintraub NL, VanRollins M, and Spector AA.** Functional implications of a newly characterized pathway of 11,12-epoxyeicosatrienoic acid metabolism in arterial smooth muscle. *Circ Res* 79: 784–793, 1996.
11. **Fang X, Moore SA, Stoll LL, Rich G, Kaduce TL, Weintraub NL, and Spector AA.** 14,15-Epoxyeicosatrienoic acid inhibits prostaglandin E_2 production in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 275: H2113–H2121, 1998.
12. **Fang X, Weintraub NL, Stoll LL, and Spector AA.** Epoxyeicosatrienoic acids increase intracellular calcium concentration in vascular smooth muscle cells. *Hypertension* 34: 1242–1246, 1999.
13. **Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, and Busse R.** Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 401: 493–497, 1999.
14. **Fitzpatrick FA, Ennis MD, Baze ME, Wynalda MA, McGee JE, and Liggett WF.** Inhibition of cyclooxygenase activity and platelet aggregation by epoxyeicosatrienoic acids. Influence of stereochemistry. *J Biol Chem* 261: 15334–15338, 1986.
15. **Fleming I.** Cytochrome p450 and vascular homeostasis. *Circ Res* 89: 753–762, 2001.
16. **Fleming I, Fisslthaler B, Michaelis UR, Kiss L, Popp R, and Busse R.** The coronary endothelium-derived hyperpolarizing factor (EDHF) stimulates multiple signalling pathways and proliferation in vascular cells. *Pflügers Arch* 442: 511–518, 2001.
17. **Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Hing HA, Gimbrone MAJ, Luster AD, Luscinskas FW, and Rosenzweig A.** MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 398: 718–723, 1999.
18. **Gordon JS, Heller SK, Kaduce TL, and Spector AA.** Formation and release of a peroxisome-dependent arachidonic acid metabolite by human skin fibroblasts. *J Biol Chem* 269: 4103–4109, 1994.
19. **Greene JF, Newman JW, Williamson KC, and Hammock BD.** Toxicity of epoxy fatty acids and related compounds to cells expressing human soluble epoxide hydrolase. *Chem Res Toxicol* 13: 217–226, 2000.
20. **Hoebel BG and Graier WF.** 11,12-Epoxyeicosatrienoic acid stimulates tyrosine kinase activity in porcine aortic endothelial cells. *Eur J Pharmacol* 346: 115–117, 1998.
21. **Huo Y, Weber C, Forlow SB, Sperandio M, Thatte J, Mack M, Jung S, Littman DR, and Ley K.** The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J Clin Invest* 108: 1307–1314, 2001.
22. **Li PL, Chen CL, Bortell R, and Campbell WB.** 11,12-Epoxyeicosatrienoic acid stimulates endogenous mono-ADP-ribosylation in bovine coronary arterial smooth muscle. *Circ Res* 85: 349–356, 1999.
23. **Mombouli JV, Holzmann S, Kostner GM, and Graier WF.** Potentiation of Ca^{2+} signaling in endothelial cells by 11,12-epoxyeicosatrienoic acid. *J Cardiovasc Pharmacol* 33: 779–784, 1999.
24. **Nithipatikom K, Pratt PF, and Campbell WB.** Determination of EETs using microbore liquid chromatography with fluorescence detection. *Am J Physiol Heart Circ Physiol* 279: H857–H862, 2000.
25. **Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, and Liao JK.** Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* 285: 1276–1279, 1999.
26. **Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, and Liao JK.** Activation of Gαs mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. *J Biol Chem* 276: 15983–15989, 2001.

27. **Oltman CL, Weintraub NL, VanRollins M, and Dellsperger KC.** Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. *Circ Res* 83: 932–939, 1998.
28. **Peri KG, Almazan G, Varma DR, and Chemtob S.** A role for protein kinase Cα in stimulation of prostaglandin G/H synthase-2 transcription by 14,15-epoxyeicosatrienoic acid. *Biochem Biophys Res Commun* 244: 96–101, 1998.
29. **Rollins BJ.** Chemokines and atherosclerosis: what Adam Smith has to say about vascular disease. *J Clin Invest* 108: 1269–1271, 2001.
30. **Shen XY, Figard PH, Kaduce TL, and Spector AA.** Conversion of 15-hydroxyeicosatetraenoic acid to 11-hydroxyhexadecatrienoic acid by endothelial cells. *Biochemistry* 27: 996–1004, 1989.
31. **Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, and Gonzalez FJ.** Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. *J Biol Chem* 275: 40504–40510, 2000.
32. **Snyder GD, Krishna UM, Falck JR, and Spector AA.** Evidence for a membrane site of action for 14,15-EET on expression of aromatase in vascular smooth muscle. *Am J Physiol Heart Circ Physiol*. (July 8, 2002). 10.1152/ajpheart.00321.2002.
33. **VanRollins M, Kaduce TL, Knapp HR, and Spector AA.** 14,15-Epoxyeicosatrienoic acid metabolism in endothelial cells. *J Lipid Res* 34: 1931–1942, 1993.
34. **Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, and Spector AA.** Potentiation of endothelium-dependent relaxation by epoxyeicosatrienoic acids. *Circ Res* 81: 258–267, 1997.
35. **Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, and Spector AA.** Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids. *Am J Physiol Heart Circ Physiol* 277: H2098–H2108, 1999.
36. **Wong PY, Lai PS, and Falck JR.** Mechanism and signal transduction of 14(R),15(S)-epoxyeicosatrienoic acid (14,15-EET) binding in guinea pig monocytes. *Prostaglandins Other Lipid Mediat* 62: 321–333, 2000.
37. **Yu Z, Xu F, Huse LM, Morisseau C, Draper AJ, Newman JW, Parker C, Graham L, Engler MM, Hammock BD, Zeldin DC, and Kroetz DL.** Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. *Circ Res* 87: 992–998, 2000.
38. **Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, and Capdevila JH.** Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *J Biol Chem* 268: 6402–6407, 1993.
39. **Zeldin DC.** Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem* 276: 36059–36062, 2001.